Degradation of Fluorobenzene by *Rhizobiales* Strain F11 via *ortho* Cleavage of 4-Fluorocatechol and Catechol[▽]

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The aerobic metabolism of fluorobenzene by *Rhizobiales* sp. strain F11 was investigated. Liquid chromatography-mass spectrometry analysis showed that 4-fluorocatechol and catechol were formed as intermediates during fluorobenzene degradation by cell suspensions. Both these compounds, unlike 3-fluorocatechol, supported growth and oxygen uptake. Cells grown on fluorobenzene contained enzymes for the *ortho* pathway but not for *meta* ring cleavage of catechols. The results suggest that fluorobenzene is predominantly degraded via 4-fluorocatechol with subsequent *ortho* cleavage and also partially via catechol.

During the last decades, environmental contamination by fluorinated organic compounds has received increasing attention because of their use as herbicides, fungicides, surfactants, refrigerants, intermediates in organic synthesis, solvents, and pharmaceuticals (11). Whereas the biodegradation of chlorinated compounds has been studied quite extensively (19), little is known about the bacterial metabolism of fluoroaromatic compounds, even though there have been several reports on the degradation of fluorobenzoic acids (5, 6, 7, 16). With chloroaromatics, most degradation routes involve dioxygenase- and dehydrogenase-mediated conversion to the corresponding chlorocatechols, which are further metabolized by a dioxygenase that cleaves the aromatic ring. Dehalogenation occurs during metabolism of the ring-cleavage products (19). Most described strains degrade chlorocatechols via the *ortho*-cleavage pathway (14, 18, 19, 20), but *meta* cleavage of 3-chlorocatechol can also occur (13), even though the *meta*-cleavage route is often unproductive due to the formation of toxic or dead-end products (1, 19). Dehalogenation may in some cases occur prior to ring cleavage. For example, mutants of *Pseudomonas* sp. strain B13 and *Alcaligenes eutrophus* B9 that grow on 2-fluorobenzoate use a dioxygenase to convert it to catechol, with concomitant decarboxylation and defluorination (5). *Pseudomonas putida* strain CLB 250, which can use three different 2-halobenzoates, also converts these substrates by initial dehalogenating dioxygenation (6), and a defluorinating 4-fluorobenzoate monooxygenase has been reported as well (16).

The present paper describes a metabolic pathway for fluorobenzene (FB). Information about the bacterial metabolism of this compound is scarce, despite studies on its chlorinated analogue (13, 18). Lynch et al. (12) described the oxidation of FB to 3-fluorocatechol by a strain of *Pseudomonas putida*, but in this study FB was not used as a carbon source. Recently, FB was reported to be completely degraded by a bacterial consortium (2) and by a pure bacterial culture that utilized it as a sole carbon and energy source (3). This gram-negative bacterium, phylogenetically classified within the order *Rhizobiales*, was named strain F11 and was used here to investigate the metabolism of FB.

Intermediates produced during FB degradation. In order to obtain information about the degradation pathway of FB, we tested which intermediates accumulated upon incubation of concentrated cell suspensions of strain F11 with FB. First, cells were grown in sealed flasks on FB in mineral medium as described previously (3), harvested by centrifugation (10,000 × *g* for 15 min at 4°C), washed twice with mineral medium, and resuspended in the same medium to give an optical density at 600 nm of 0.3. Glucose (1 mM) was added, since it was found in preliminary experiments that this enhanced degradation of FB and stimulated accumulation of intermediates. The suspensions were incubated in closed flasks with FB, and samples were taken at appropriate times, centrifuged, and subjected to high-performance liquid chromatography (HPLC), gas chromatography, and fluoride measurements. Fluoride was measured with a Dionex Dx-120 ion chromatograph equipped with an Allsep A-2 anion column from Alltech, and the eluent was a mixture of $NaHCO_3$ and Na_2CO_3 in deionized water. For FB analysis, culture samples were extracted with diethylether and analyzed by gas chromatography as described previously (3). It was observed that whole cells of strain F11 completely removed 1.1 mM FB in 13 h, but stoichiometric fluoride release was seen only after 29 h (Fig. 1). This indicates that fluorinated intermediates did temporarily accumulate but that there was no formation of high levels of fluorinated dead-end metabolites.

Samples (20 μl) from the same culture fluid were also analyzed by HPLC and liquid chromatography-mass spectrometry (LC-MS). HPLC analysis was carried out on a Lichrospher 100 RP8 reversed-phase column in connection with Jasco PU-980 pumps, a Jasco MD-910 diode array detector, and a Jasco UV-2075 detector. Compounds were isocratically eluted at a

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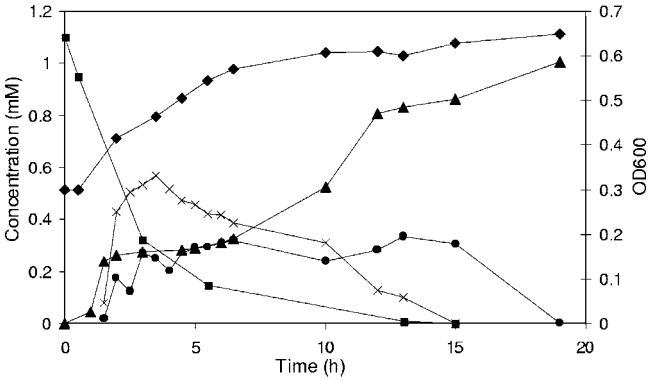

FIG. 1. Accumulation of metabolites during degradation of 1.1 mM FB by strain F11. Symbols: ■, FB (calculated as if all substrate was present in the liquid phase); ▲, fluoride; X, 4-fluorocatechol; ●, catechol; ◆, optical density at 600 nm. Liquid phase FB is in equilibrium with gas-phase substrate according to the Henry coefficient ($H = C_g/C_l = 0.258$). The closed flasks had a volume of 1,200 ml and contained 250 ml medium.

flow rate of 1 ml/min with a solution of water-acetonitrile (80:20) and 10 mM formic acid. LC-MS was carried out with a Micromass ZMD detector equipped with a XTerra MS, a SymmetryShield C8 column (4.6 mm by 150 mm), a 996 photodiode array detector, and a 2690 separations module, all from Waters. While degradation proceeded, five metabolites (compounds I to V) appeared in the culture medium (Table 1). Four of these (compounds I, II, IV, and V) were completely consumed during prolonged incubation. One minor metabolite (compound III) remained in the culture supernatant even after incubation for 48 h.

Metabolite I, which appeared early, coeluted with catechol and had a molecular mass (negative-mode MS) of $m/z = 109$ ($M\text{-}H^+$). Metabolite II was identified as 4-fluorocatechol on the basis of cochromatography with a standard in HPLC analysis and its negative-mode mass spectrum with $m/z = 126.91$ ($M\text{-}H^+$). The dead-end metabolite III was identified as *cis*-dienelactone by cochromatography and mass spectrometry (positive ionization, $m/z = 140.04$ [$M+H^+$]). Metabolites IV and V could not be identified, since no ionization was obtained with LC-MS.

The occurrence of the two catechols during the initial 20 h of FB degradation (Fig. 1) suggests that strain F11 converts FB partially to catechol and partially to 4-fluorocatechol during the first metabolic step. Approximately 0.6 mM of the FB that was converted transiently appeared as 4-fluorocatechol, and about 0.3 mM was detected as catechol. This is in agreement with the observation that significant fluoride release is taking place already during the initial period of FB degradation, i.e., when catechol is formed but that fluoride release is only complete when the intermediate 4-fluorocatechol and possibly other fluorinated metabolites have been degraded. The formation of both catechol and 4-fluorocatechol from FB is also consistent with the ability of strain F11 to grow on both of these catechols. The fact that catechol remained in the medium for quite a long time even though it is a better growth substrate than 4-fluorocatechol suggests inhibition of the catechol pathway by the presence of 4-fluorocatechol.

TABLE 1. HPLC retention times and absorption maxima of metabolic intermediates formed by F11 cells exposed to fluorobenzene in the presence of glucose

Compound	Retention time (min)	λ_{max} (nm)	Growth in substrate[a]
Catechol (metabolite I)	8.4	195, 276	+
4-Fluorocatechol (metabolite II)	13	190, 280	+
cis-Dienelactone (metabolite III)	8	190, 220, 275	NT
Metabolite IV	23.4	273	NT
Metabolite V	6	210	NT
Fluorobenzene	65	260	+
3-Fluorocatechol	10.5	190, 267	−
cis,*cis*-Muconic acid	6.25	260	+
Phenol	15	270	+
4-Fluorophenol	22	190, 277	+
Hydroquinone	5.2	280	NT
1,2,4-Benzenetriol	4	280	NT

[a] Abbreviations: NT, not tested; −, no growth after 14 days; +, clearly visible growth in liquid culture after 4 days.

Substrate-dependent oxygen consumption by whole cells. In order to test the inducibility of FB transformation activity, oxygen uptake measurements were done. Cells of strain F11 were grown on FB, benzene, or citrate, harvested by centrifugation, washed, resuspended to a density of 0.43 mg of cellular protein per ml, and transferred to a stirred vessel that was equipped with a fiber optic oxygen sensor (MOPS-1; ProSense BV, Hanover, Germany). The rate of O_2 consumption was measured at room temperature in the presence of different substrates (Table 2).

Cells grown on FB oxidized fluorobenzene and *cis*-1,2-dihydrobenzenediol as well as 4-fluorocatechol and were also highly induced for the oxidation of catechol. Rapid oxidation of catechol, 4-fluorocatechol, and *cis*-1,2-dihydrobenzenediol was also obtained with cells that were pregrown on benzene. With fluorobenzene and benzene, these cells showed even higher oxygen uptake rates than cells grown on FB. With cells grown on citrate, the aromatic substrates were not oxidized, suggesting that the formation of the first catabolic enzyme was induced during growth on the aromatic substrates and repressed on citrate. The oxygen uptake rates with 3-fluorocatechol were very low, independent of whether the cells were grown on FB, benzene, or citrate. The patterns of oxygen

TABLE 2. Substrates oxidized by fluorobenzene-, benzene-, and citrate-grown cells of strain F11[a]

Assay substrate	Rate of oxygen consumption (nmol/min per mg of cells) after growth with:		
	Fluorobenzene	Benzene	Citrate
Fluorobenzene	66	420	<0.1
Benzene	2	220	<0.1
cis-1,2-Dihydrobenzenediol	160	400	<0.1
Catechol	570	340	<0.1
3-Fluorocatechol	<0.1	<0.1	<0.1
4-Fluorocatechol	300	570	<0.1
Citrate	<0.1	<0.1	151

[a] Oxygen consumption was measured with an oxygen sensor as described in Materials and Methods. All substrates were used at a concentration of 1 mM. Results represent the means of the results of at least three independently performed experiments. Oxygen uptake experiment results are corrected for endogenous respiration; replicates showed less than 10% variation.

TABLE 3. Enzyme activities in cell extracts of strain F11 grown on FB

Enzyme	Assay substrate	Sp act[b] (U · mg of protein^{-1})
Catechol 1,2-dioxygenase	Catechol	0.96
	3-Fluorocatechol[a]	0.009
	4-Fluorocatechol	0.16
Catechol 2,3-dioxygenase	Catechol	0.002
Muconate cycloisomerase	*cis*,*cis*-Muconic acid	0.200
Dienelactone hydrolase	*cis*-Dienelactone	0.002
Maleylacetate reductase	Maleylacetate	0.78
3-Oxoadipate:succinyl-CoA transferase	3-Oxoadipic acid	0.12

[a] Activity with 3-fluorocatechol was tested both at 0.5 and 0.1 mM; the results obtained were always very similar.

[b] Numbers represent the means of the results of at least three independently performed experiments.

consumption show that oxidation of catechol, 4-fluorocatechol, and *cis*-1,2-dihydrobenzenediol was induced by FB and benzene, whereas 3-fluorocatechol was never oxidized. When batch cultures of strain F11 growing with 1 mM of FB were supplemented with 0.1 mM of 3-fluorocatechol, FB was no longer converted by the cells, and fluoride, measured with a fluoride-selective electrode (2), was not released.

Enzymes involved in the degradation of FB. To investigate whether degradation of FB proceeds via *meta* or *ortho* cleavage, the presence of several enzymes involved in these routes was tested (Table 3). Late exponential FB-grown cells were harvested by centrifugation, washed twice with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 mM 1,4-dithiothreitol, and disrupted by sonication in the same buffer. After centrifugation (90,000 × *g* for 60 min at 4°C) the clear supernatant was used as the cell extract for enzyme assays. Its protein content was determined with Coomassie brilliant blue using bovine serum albumin as the standard.

Catechol 2,3-dioxygenase was measured by determining the formation of 2-hydroxymuconic semialdehyde (ε = 44,000 M^{-1} cm^{-1}) at 375 nm, according to the method of Nozaki (15). (Fluoro) catechol 1,2-dioxygenase activity was measured similarly, as described by Dorn and Knackmuss (4) ($\varepsilon_{cis,cis\text{-muconate}}$ = 16,800 M^{-1}, $\varepsilon_{2\text{-fluoro-}cis,cis\text{-muconate}}$ = 14,900 M^{-1} cm^{-1}, $\varepsilon_{3\text{-fluoro-}cis,cis\text{-muconate}}$ = 14,900 M^{-1} cm^{-1}). Muconate cycloisomerase activity was measured by following the consumption of *cis*,*cis*-muconate in an assay mixture containing 30 mM Tris-HCl (pH 8.0), 1 mM $MnCl_2$, and 0.1 mM *cis*,*cis*-muconate. Dienelactone hydrolase activity was determined by following at 280 nm (ε = 17,000 M^{-1} cm^{-1}) the decrease in the level of 0.1 mM *cis*-dienelactone that was incubated with enzyme in 10 mM histidine-HCl (pH 6.5). Maleylacetate was prepared on the day of its use by alkaline hydrolysis of *cis*-dienelactone (8), and the reductase was measured by following maleylacetate (0.1 mM)-dependent NADH (0.2 mM) oxidation at 340 nm in 50 mM Tris-HCl (pH 7.5). Activities were corrected for substrate-independent NADH oxidation. 3-Oxoadipate:succinyl-coenzyme A transferase was measured as described by Mars et al. (13). One unity of activity was defined as the amount of enzyme required to convert 1 μmol of substrate per min at 25°C.

Activities of the *ortho* pathway enzymes catechol 1,2-dioxygenase, muconate cycloisomerase, maleylacetate reductase, and 3-oxoadipate:succinyl-coenzyme A transferase were found in cell extracts of strain F11 grown on FB. Catechol 2,3-dioxygenase activity was not detected, indicating that strain F11 does not use a *meta*-cleavage pathway to degrade FB. A 1,2-dioxygenase activity was detected with both catechol and 4-fluorocatechol but was hardly detected with 3-fluorocatechol. Instead, the catechol 1,2-dioxygenase activity was reduced by 70% and 90% in the presence of 0.1 and 0.5 mM 3-fluorocatechol, respectively. A muconate cycloisomerase activity with respect to *cis*,*cis*-muconic acid was also detected. No activity was found for *cis*-dienelactone hydrolase in extracts of strain F11. These observed enzyme activities suggest that the catechols in the FB degradation pathway undergo *ortho* cleavage.

We judge it highly unlikely that 3-fluorocatechol is an intermediate in FB degradation pathway, since it was not used as a growth substrate and it strongly inhibited FB degradation and growth and since no dioxygenase activity with 3-fluorocatechol was detected in cell extracts of strain F11 grown on FB. This conclusion is in line with the previously described resistance of the expected product 2-fluoro-*cis,cis*-muconic acid to enzymatic cycloisomerization (23), although it is risky to generalize such a finding to other organisms. Furthermore, in mutants of *Alcaligenes eutrophus* B9 and *Pseudomonas* sp. strain B13 that use 2-fluorobenzoate for growth, the formation of toxic 3-fluorocatechol is prevented by loss of dihydrodihydroxybenzoate dehydrogenase activity, allowing growth on catechol that can be formed by initial dioxygenation of the aromatic ring (5). The absence of a 3-halocatechol intermediate clearly distinguishes the fluorobenzene pathway of strain F11 from chlorobenzene catabolic pathways, which proceed via 3-chlorocatechol (13, 17, 18). This could explain the lack of growth of strain F11 on chlorobenzene.

Pathway of FB degradation. We propose the pathway for FB metabolism shown in Fig. 2. The initial attack of FB by dioxygenase activity yields two different fluorinated dihydrodiols. The 4-fluoro-*cis*-benzene-1,2-dihydrodiol that is produced is transformed into 4-fluorocatechol by a dihydrodiol dehydrogenase. Conversion of the other product, 1-fluoro-*cis*-benzene-1,2-dihydrodiol, to catechol can proceed without involvement of dehydrogenase that reduces a cofactor, since the electrons are transferred to the fluoride that is being released (Fig. 2). Simultaneous conversion of a fluorinated compound to catechol and a fluorinated catechol was described earlier for the degradation of 2-fluorobenzoate by *Pseudomonas* sp. strain B13 and strain FLB300 (5, 7).

The capability of strain F11 cells to use 4-fluorocatechol as a growth substrate, its transient accumulation in cell suspensions to higher levels than catechol, and the fact that 4-fluorocatechol stimulated oxygen uptake by whole cells lead to the conclusion that 4-fluorocatechol is the predominant intermediate. The occurrence of 4-fluorocatechol as an intermediate has also been described for the aforementioned α-proteobacterium strain FLB300, which degrades both benzoate and all monofluorosubstituted benzoates (7). The metabolism of 4-fluorocatechol is proposed to proceed through *ortho* cleavage by a (fluoro)catechol 1,2-dioxygenase that yields 3-fluoro-*cis*,*cis*-muconate. This product could be transformed with concomitant defluorination into maleylacetate via either 4-fluoromuconolactone or another lactone derivative. *ortho* cleav-

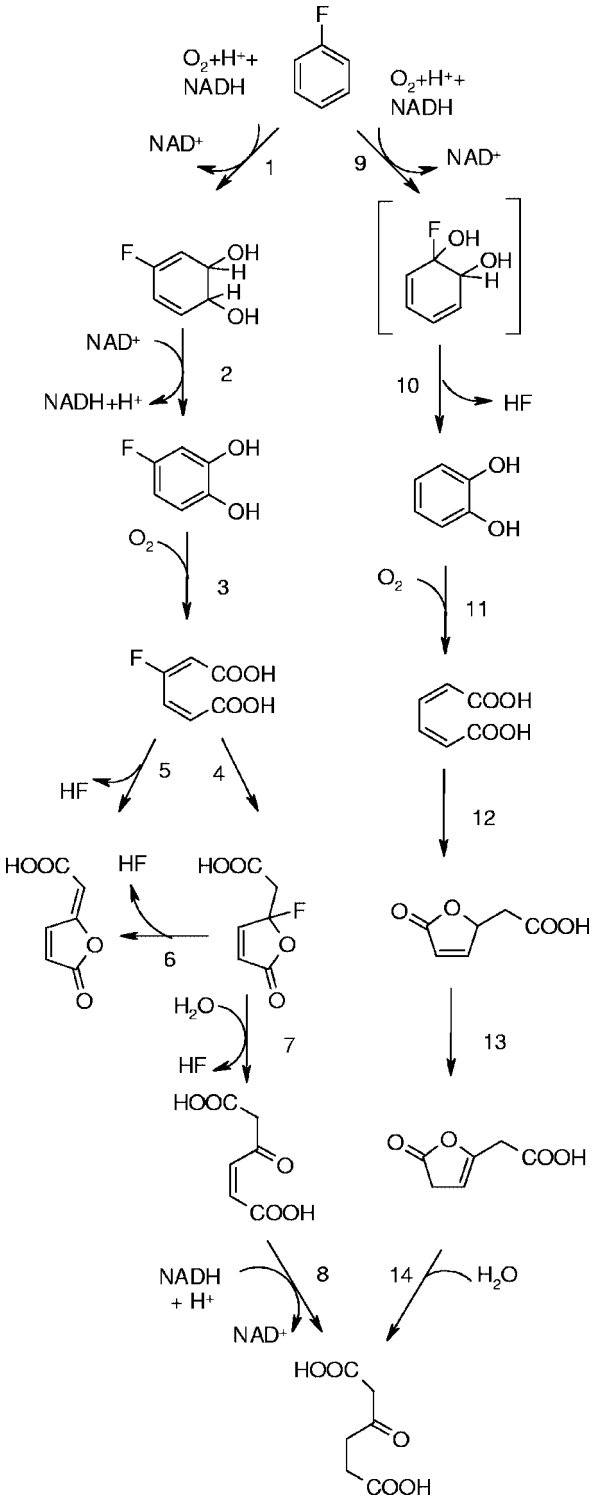

FIG. 2. Proposed pathway for fluorobenzene metabolism by strain F11. The enzyme activities are denoted as follows: 1, fluorobenzene dioxygenase; 2, fluorobenzene dihydrodiol dehydrogenase; 3, fluorocatechol 1,2-dioxygenase; 4, fluoromuconate cycloisomerase; 5 and 6, possible side reactions to *cis*-dienelactone by fluoromuconate cycloisomerase (activity 5) or by slow spontaneous conversion (activity 6); 7, *trans*-dienelactone hydrolase; 8, maleylacetate reductase; 9, fluorobenzene dioxygenase; 10, nonenzymatic defluorination; 11, catechol 1,2-dioxygenase; 12, muconate cycloisomerase; 13, muconolactone isomerase; 14, 3-oxoadipate enol-lactone hydrolase.

age of 4-fluorocatechol is also a key step in the metabolism of 3- and 4-fluorobenzoate by several bacterial strains (9, 21, 22, 24). These convert 4-fluorocatechol via 3-fluoro-*cis*,*cis*-muconate and 4-fluoromuconolactone (22, 24) or via a non-fluorinated dienelactone intermediate (24). If 4-fluorocatechol metabolism in strain F11 proceeds in the same way, this could also explain the accumulation of *cis*-dienelactone since it can slowly be formed as a side product during spontaneous or enzyme-catalyzed dehydrodefluorination of 4-fluoromuconolactone (23, 24). This pathway would also allow formation of protoanemonin.

Maleylacetate can be channeled into the tricarboxylic acid cycle via 3-oxoadipate. Catechol, the minor product of the initial dioxygenation reaction, is proposed to be metabolized to *cis,cis*-muconate, converted to the lactone derivative, and then also channeled into the 3-oxoadipate route (Fig. 2).

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